Mucus and mucins during gastrointestinal infections

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Gothenburg 2014

Cover illustration: *PAS/Alcian blue staining of an intestinal in vitro mucosal surface*

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ISBN 978-91-628-8942-5
http://hdl.handle.net/2077/34844
Gothenburg, Sweden 2014
Printed by Ineko

To Sohrab, the meaning of my life
and my inspiration

ABSTRACT

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The gastrointestinal tract is protected by a continuously secreted mucus layer formed by mucin glycoproteins. The mucus layer and mucins change dynamically during infection. The main focus of this thesis was to investigate the changes in mucin and the mucus layer in the gastrointestinal tract during infection with the gastrointestinal pathogens *C. rodentium* (a mouse model for intestinal A/E pathogens), ETEC and *H.pylori*. To be able to compare the results from murine studies to the effect of infection in humans, we needed an *in vitro* mucosal surface to most resemble the *in vivo* environment. Therefore, we developed a method of culture to create an *in vitro* model suitable for studies of host-pathogen interactions at the mucosal surface that caused the cells to polarize, form functional tight junctions, a three-dimensional architecture resembling colonic crypts, and produce an adherent mucus layer.

We investigated the effect of infection with *H. pylori* on mucin synthesis *in vivo*. The results of our non-radioactive "pulse" experiments showed *H. pylori* colonization in the mucus niche of the murine stomach leads to decreased mucin production and secretion rate. *H. pylori* infection also decreased levels of MUC1 in the mucosa.

The effect of *C. rodentium* infection on the distinct aspects of the mucus layer and mucins was also investigated during this work. Our results in the WT mice demonstrated mucus transcription and secretion are dynamically altered in response to the infection. Furthermore, the clearance of the infection coincides with the reformation of the organized inner mucus layer and an increased mucus thickness, which corresponded with altered ion channel activities.

To examine the effect of the cytokine environment on the changes of mucin and mucus layer, we infected WT and IFN-$\gamma^{-/-}$ mice with *C. rodentium* that resulted in a vast enhancement of mucus thickness in the IFN-$\gamma^{-/-}$ mice compared to the WT animals. The effect of individual cytokines was further studied using our *in vitro* model with and without infection with *C. rodentium*/ETEC. The outcome demonstrated that changes in the goblet cells, mucin and mucus layer during infection is dependent on the combined impact of the pathogen and cytokines, and that the presence of the Th2 cytokines accelerated the process of mucin synthesis.

Keywords: *Mucin, gastrointestinal cell lines, mucus layer, secreted mucin, cell surface mucin, H. pylori, C. rodentium, ETEC, mucin secretion, goblet cells*

ISBN: 978-91-628-8942-5
http://hdl.handle.net/2077/34844

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals:

I. **Helicobacter pylori infection impairs the mucin production rate and turnover in the murine gastric mucosa.**
Navabi N, Johansson ME, Raghavan S, Linden SK (2013), Infect Immun 81: 829-837.

II. **Gastrointestinal cell lines form polarized epithelia with an adherent mucus layer when cultured in semi-wet interfaces with mechanical stimulation.**
Navabi N, McGuckin MA, Linden SK (2013), PLoS One 8: e68761.

III. **Dynamic Changes in Mucus Thickness and Ion Secretion during Citrobacter rodentium Infection and Clearance.**
Gustafsson JK*[1], Navabi N*, Rodriguez-Pineiro AM, Alomran AH, Premaratne P, Fernandez HR, Banerjee D, Sjövall H, Hansson GC, Lindén SK (2013), PLoS One 8: e84430.

IV. **Impact of cytokine environment on mucins during infection wih intestinal pathogens.**
Navabi N, Gustavsson J, Sjöling Å, Lindén SK (2014).
Manuscript

[1] *These authors contributed equally to this work.

CONTENT

ABBREVIATIONS

A/E	Attaching and effacing
C. rodentium	*Citrobacter rodentium*
Cag A	Cytotoxin-assocciated gene A
cAMP	Cyclic AMP
CCh	Charbacol
Cp	Membrane capacitance
CT	Cholera toxin
E. coli	*Escherichia coli*
EHEC	Enterohemorrhagic *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
GI	Gastrointestinal
H. pylori	*Helicobacter pylori*
H. pylori SS1	*H. pylori* (Sydney strain)
IFN	Interferon
IFN-$\gamma^{-/-}$	Interferon deficient
IL	Interleukin
LEE	Locus of enterocyte
Muc2$^{-/-}$	MUC2 deficient
PD	Transepithelial potential
Rp	Epithelial resistance
TNF	Tumor necrosis factor
Th	T helper cell
Vac A	Vacuolating cytotoxin
WT	Wild-type

INTRODUCTION

Background

The mucosal tissues of the gastrointestinal tract present an enormous surface area to the exterior environment, and are frequently challenged by pathogens and antigens contained in food and water. In fact, mucosal tissues represent the site of infection or route of access for the majority of bacteria that cause human disease and are major sources of morbidity and mortality. One very prevalent gastrointestinal infection is diarrheal disease, which is the third most common cause of mortality from infectious diseases worldwide. Gastrointestinal infection is also associated with chronic diseases, such as *Helicobacter pylori* infection, which can lead to gastric cancer. Half the world population is estimated to be infected with *H. pylori* [1].

The mucus layer that covers the mucosal tissue of the gastrointestinal tract is the first barrier that encounters the pathogens, and it is continually washing the mucosal surfaces and removing trapped bacteria. However, there are studies that indicate different bacterial species have gained the ability to alter the features and dynamics of mucins, which are the main component of mucus, and colonize the mucosal surface. At present, the mechanism of interaction between pathogens and mucus during infection is not completely characterized. Since the bacterial penetration into the mucus layer and colonization is the first step in the establishment of infectious diseases, a characterization of the regulatory networks that interface with mucin producing cells may have broad biomedical application.

The gastrointestinal tract

The gastrointestinal (GI) tract is a system of organs that facilitates the ingestion, digestion, and absorption of food with the subsequent defecation of waste [1]. It consists of the oral cavity, esophagus, stomach, small intestine, large intestine, and the anus. The GI tract is divided into four concentric layers: mucosa, submucosa, muscularis externa, and adventitia or serosa [2]. The mucosa is the innermost layer surrounding the lumen and consists of epithelium, lamina propria, and muscularis. The self-renewing epithelial cells of the mucosal surface secrete mucins, enzymes, and other biochemicals that either aid in digestion or protect the mucosa [2]. The gastric epithelium contains multiple cell lineages and is organized into four distinct zones, surface/pit foveolus, isthmus, neck and base, and its mucin producing cells consist of surface, neck and pit cells [1]. The intestinal epithelium mainly consists of

four different cell types: absorptive (enterocytes), enteroendocrine, mucosecreting (goblet cells) and Paneth cells [2].

The Mucosal Surface

The mucosal surface of the GI tract is the first barrier that protects this organ against potential insults from the environment. The importance of the defense mechanism of this surface is highlighted by the fact that gastrointestinal infections kill around 2.2 million people globally each year [2]. The front line of this protective barrier is the mucus layer that covers the epithelial cells and acts as a lubricant, facilitate absorption of nutrients, provide a favorable environment for commensal microorganisms, occlude microbial invasion and provide a matrix for a broad spectrum of antimicrobial molecules [3,4]. The thickness of the mucus layer varies throughout the GI tract and is thickest in the colon and stomach and thinnest in the jejunum [3,4]. The continuous mucus layer in the stomach and colon consists of two layers: an adherent inner layer that covers the mucosa, and a thicker loose layer. The inner layer of the colon is densely packed, and has a compact and stratified appearance. The small pore size of this layer can physically prevent bacterial penetration; therefore, this inner layer is normally devoid of bacteria [5]. In contrast, the inner layer of the stomach is fully penetrable by beads the size of bacteria [6]. Furthermore, it maintains a pH gradient ranging from 2 at the lumen and 7 at the epithelial surface [7]. The inner layer is well organized with a laminated appearance, and converts into the outer layer. The outermost loosely adherent mucus layer is continually removed by movement of the luminal contents and renewed, which results in clearing trapped material. In contrast to stomach and colon, the small intestine contains one loose layer of mucus, which is permeable to bacteria [5].

Underneath the mucus layer, a second line of defense is comprised by the epithelial glycocalyx, which is partially integrated with the overlying mucus gel. The glycocalyx is an extracellular zone on the apical surface of mucosal epithelial cells, composed of proteoglycans, glycolipids and transmembrane glycoproteins including cell-surface mucins [8]. Some definitions of glycocalyx include the mucus layer, however, in this thesis we refer to glycocalyx and the mucus layer as separate entities. The glycocalyx also varies in thickness depending on body location [8].

Mucins

The main components of the mucus layer are mucin glycoproteins produced by epithelial mucus-producing cells, including goblet cells. Mucins are also a major part

of the glycocalyx. They are highly glycosylated glycoproteins consisting of a protein backbone (apomucin) with a few *N*-glycan chains and large number of relatively short oligosaccharides side chains attached to serine (Ser) or threonine (Thr) residues via *O*-glycosidic linkages [9]. Mucin domains are built on a protein core that is rich in the amino acids proline, serine and threonine (called PTS sequences). There is genetic polymorphism in these sequences which is known as "variable number of tandem repeats" (VNTR), although as lately shown, this is not always the case [10]. The oligosaccharides chains that are clustered into the highly glycosylated domains are the cause of the "bottle brush" appearance of mucins. These structures are important in covering the protein backbone and protect the mucins from digestion and bacterial proteases [11]. The chains are terminated by fucose, galactose, GalNAc or sialic acid residue in the peripheral region that can form histo-blood-group antigens such as A, B, H, Lewis a (Le^a), Lewis b (Le^b), Lewis x (Le^x), Lewis y (Le^y), sialyl-Le^a and sialyl-Le^x structures [12]. The *N*-linked oligosaccharides are associated with folding and oligomerization or surface localization [13,14]. In general, mucins are divided into: secreted (gel-forming), secreted (non-gel forming) and cell-surface mucins [15].

Secreted mucins

The secreted mucins are gel-forming except for MUC7 (found in saliva), and are responsible for the viscous property of the mucus layer. In the stomach, MUC5AC produced by surface epithelial cells and MUC6 secreted from sub-mucosal glands are the major secreted mucins, while intestinal goblet cells secret MUC2, the predominant mucin in the intestine. Genes of these secreted gel-forming mucins are clustered on chromosome 11p15 and share some sequence homology [16]. The core proteins of secreted mucins are very large (typically more than 5,000 amino acids), and their ability to form mucin-type gels results from polymerization of their C- and *N*-terminal regions that contain conserved cysteine-rich von Willebrand D domains [17].

Synthesis of secreted mucin

The assembly pathway of mucin synthesis in the highly polarized mucin producing cells start at the endoplasmic reticulum (ER), in the basal cytoplasm of these cells. The protein backbones of the mucins are synthesized on polyribosomes, located at the cytoplasmic face of the ER, and co-transitionally translocated across the membrane into the lumen of the ER. During this translocation, *N*-glycosylation of mucin monomers occurs in the polypeptide chain followed by intramolecular disulfide-stabilized folding. Intermolecular disulfide bonds between the monomers, mediated by cysteine residues in the C-terminus (cysteine knot domain), results in

non-*O*-glycosylated dimmers. Correct folding of the protein is essential at this point to allow the translocation of mucin dimers to the Golgi complex and prevent their aggregation in the ER [18-20]. This translocation occurs by vesicular transport of the mucin to the Golgi where *O*-glycan chains are attached to the mucin by glycosyltransferases [15]. The *O*-glycan elongation is dependent on the initial addition of GalNAc to serine and threonine residues, and this key step of the process is catalyzed by polypeptide-GalNAc transferases. After glycosylation, while transiting through the trans-Golgi network, the dimers are further polymerized [21], and are packed into secretory granules and stored [22,23]. The exact mechanism of this packaging into a highly condensed and dehydrated state in the secretory granules is not completely clear. However, pH and Ca^{2+} ions have been implicated as essential components; calcium ions being particularly important for shielding the negatively charged sugars [24-26]. A summary of the process of mucin synthesis is shown in the schematic picture in Figure 1A.

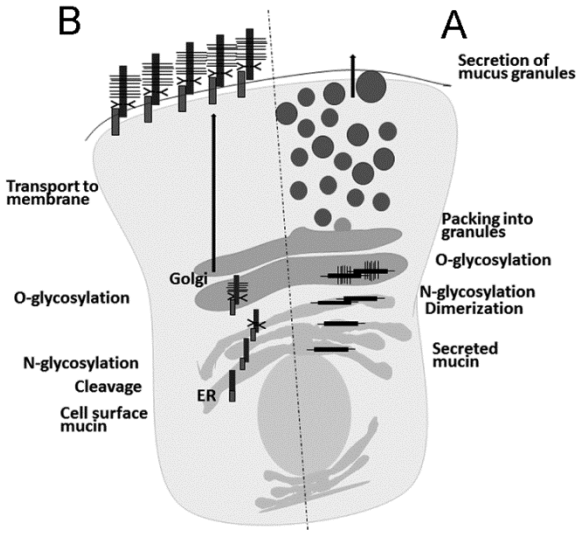

Figure 1. Schematic of cell-surface and secreted mucins biosynthesis in the epithelial cells. The schematic shows synthesis of A) secreted mucin in goblet cells, B) cell-surface mucins in epithelial cells. In the ER, secreted mucins are *N*-glycosylated and dimerize via their C-terminal domains. Cell-surface mucins are cleaved into two subunits in the endoplasmic reticulum, inserted into the membrane and N-glycosylated. Both cell surface and secreted mucins are *O*-glycosylated in the Golgi. Following completion of *O*-glycosylation, the dimers of the secreted mucins undergo *N*-terminal oligomerization and are packed into granules for secretion.

Ion channels and mucin release

Post-secretion, the mucins mature by quick release and hydration that result in more than a 1000 fold expansion [27]. Bicarbonate ions in the extracellular milieu are suggested to be the factor that is involved in removing Ca^{2+} ion and increasing pH [28]. The importance of Ca^{2+} ions in the release of mucin is supported by the studies on MUC2 [26,27,29]. Bicarbonate is exchanged for chloride or short-chain fatty acids [30] and its secretion is modulated by a variety of secretagogues that can induce cyclic AMP (cAMP) or Ca^{2+} dependent pathways. The cAMP dependent secretion opens cAMP gated K^+ channels, activates $Na^+/K^+/Cl^-$ co-transporters [31], and transports bicarbonate over the basolateral membrane by sodium-bicarbonate co-transporters (i.e. NBCel and NBCn1) [32]. The transporter for the Ca^{2+} dependent pathway relies on the bicarbonate in combination with Na^+/H^+ exchange [33] that results in increased levels of Ca^{2+} and activation of protein kinase C [34]. The exit of bicarbonate over the apical membrane has also been related to two pathways; the cystic fibrosis transmembrane conductance regulator (CFTR) channel on enterocytes, and Bestrophin-2 (Best2) (a Ca^{2+}-activated Cl channels) expressed by goblet cells [35] which are respectively activated by cAMP and Ca^{2+} pathways respectively. The activation of each cascade is dependent on the secretogogues.

Cell-surface mucins

The cell-surface mucins represent major components of the glycocalyx, and are expressed on the apical membrane of all mucosal epithelial cells. MUC1 is the main cell-surface mucin in the stomach, whereas MUC3A, MUC3B, MUC4, MUC12, MUC13, MUC15 and MUC17 are those present in the intestine [8]. Many gastrointestinal cells express multiple members of the cell-surface family, and there is a variation in the relative expression of these mucins between distinct cell types and different regions of the GI tract [36]. An integral transmembrane domain binds these mucins to cells, and their relatively short cytoplasmic tails associate with cytoskeletal elements, cytosolic adaptor proteins and/or participate in signal transduction [37]. Centrally located PTS regions are the characteristic feature of all mucins, differentiating them from other membrane-bound glycoproteins. The cell-surface mucins also have SEA domain [38] and epidermal growth factor domains [39] that are involved in cleavage and signaling pathway respectively [40,41]. The EGF-like domains are postulated to function by allowing cell-surface mucins to interact with members of the EGF receptor family, which are likely involved in regulation or signaling of growth, motility, differentiation, and inflammation [40,41]. Specific proteolytic cleavage occurs within the SEA domain of the extracellular juxtamembrane part of the cell-surface mucins during the intracellular post translational process for the part of the protein that is destined to be expressed on the

extracellular surface [42]. The two subunits created by this cleavage remain non-covalently linked during cellular transport through the endoplasmic reticulum, the Golgi complex and at the cell surface. These subunits facilitate the release of the extracellular domain at the cell surface in response to alterations in pH, ionic concentration, hydration and/or specific proteases mediators. The mechanism that controls this release has not been clearly elucidated [43]. A summary of the process of cell-surface mucins synthesis is shown in the schematic in Figure 1B.

Mucin dynamics

The mucosal barrier is not a static surface, as continuous mucus production and secretion are required to keep the homeostasis of this surface. The secretion of mucins is divided into two forms: constitutive or baseline and stimulated or accelerated secretion in response to external stimuli. The constitutive continuous secretion is necessary to maintain the thickness of mucus lost in the movement of luminal contents and/or degraded by luminal bacteria. The secretion of mucin is highly regulated by the luminal microenvironment, neural, endocrine and immune factors, and involves the transport of granules via actin remodeling, binding to the membrane and exocytosis [44,45].

Accelerated secretion is a receptor-mediated event, in which the intracellular Ca^{2+} ion level has been shown to act as the regulator [46,47]. Exposure of mucin producing cells to potent secretagogues induces a rapid increase in the release of mucin granules. The discharge occurs via fusion of the centrally stored mucin granules with the plasma membrane followed by the release of their contents into the lumen [48]. The process is continued by the fusion of adjacent vesicles to the membrane of previously released granules, and can result in release of entire mucin granule content within a minute [48]. Accelerated secretion occurs in response to a range of environmental stimuli including microbial components, inflammatory cytokines, prostaglandins, cholinergic stimuli, lipopolysaccharide, bile salts, nucleotides, nitric oxide, vasoactive intestinal peptide, neutrophil elastase, and components of the immune system [49-58]. The effect of microbial components on accelerated secretion has been detected in both *in vitro* and *in vivo* studies. For example, the cholera toxin (CT) from *Vibrio cholerae*, was shown to trigger a massive mucin release via a cAMP-dependent mechanism from cultured intestinal HT29 goblet cells [59]. *In vivo* studies in rabbit, suggested that the acceleration of mucin secretion by CT is an indirect mechanism that might be mediated by mucosal nerve cells or other cell types [60,61]. Moreover, infection of a gerbil model of amebic colitis with the enteric protozoan parasite *Entamoeba histolytica* resulted in depletion of stored mucin from the goblet cells that has been related to the effect of parasite-derived secretagogues

[62,63]. Bioactive factors of the immune system are other important stimulators of accelerated mucin secretion [45].

The mucosal immune system and mucin secretion

The mucosal immune system of the GI tract forms one of the largest immunological organs with a dual function: to protect the GI tract from invading pathogens and to tolerate both ingested food antigens and the commensal microbiota. In addition to mucins, epithelial cells secrete a number of factors that contribute to barrier function, including antimicrobial peptides, and trefoil factors. The epithelial cells are also involved in mediating the innate immune response via their pattern recognition receptors, such as Toll-like receptors, which are the nucleotide-binding oligomerization domain receptors-like receptor proteins that can identify pathogen-associated molecular patterns (PAMPs). The outcome of the induction of innate immunity is a diverse response that includes the release of anti-microbial peptides and cytotoxic molecules, phagocytosis and intracellular killing of microbes and complement activation. Furnthermore, release of innate cytokines: interleukin 1 β (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor-α (TNF-α), and release of acute-phase proteins from the liver that assist in pathogen clearance [64]. In addition, cell-surface mucins, as shown for MUC1, can modulate the activation of nuclear factor-κB (NF-κB), which is the transcriptional factor involved in the regulation of inflammatory signaling [65,66]. Underneath the epithelial layer, are the lamina propria, which are populated by immune cells, including mononuclear phagocytes (macrophages and dendritic cells) and eosinophils (in normal intestine), antibody-secreting B cells (mainly secrete IgA) and cytotoxic ($CD8^+$) and helper ($CD4^+$) T cells [64,67]. $CD4^+$ T cells can be further subdivided based on their secreted cytokines: Th1 that secretes interferon gamma (IFN-γ) and assists in clearing intracellular pathogens by activating macrophages; Th2 that secretes interleukin-4 (IL-4), interleukin-5 (IL-5), and interleukin-13 (IL-13), and provides help to B cells for antibody production; Th17 secretes interleukin-17 (IL-17), and plays an important role in microbial clearance and a destructive role in autoimmunity [49].

Stimulation of the innate and adaptive immune response, in addition to direct regulation of epithelial cells, can affect the secretion rate of mucins and differentiation of goblet cells. For example, the stimulation of the human gastric cell line with IFN-γ prompted secretion of mucus and expression of MUC6 [68]. IL-4 and IL-13 also induced the expression of the transcription factor SPDEF, which regulates goblet cell differentiation and expression of genes involved in mucin synthesis and secretion [69-73]. The decrease in the total amount of mucins in response to IFN-γ and TNF-α was detected in some experiments: simultaneous addition of IFN-γ and TNF-α to a monolayer of cultured goblet cell line Cl.16E devoided the cells of mucus

granules [74]. Vaccination of mice that induced the IFN-γ secreting cells led to a decreased level of Alcain blue/PAS staining (mucins) in the airway epithelium of mice with allergic inflammation [75]. However, an increase in mRNA of secreated mucins was detected in response to different cytokines. For example, *in vitro* stimulation of dog bronchoalveolar lavage cells with IL-9 increased MUC5AC gene expression [76]. TNF-α and IL-6 stimulated mucin secretion by cultured colonic cells and increased the gene expression of MUC2, MUC5AC, MUC5B, and MUC6 [50]. IL-1 stimulation of mucin release has been detected in rat colon [77], and the addition of this cytokine to cultured colonic LS180 cells increased the mRNA levels of MUC2 and MUC5AC [50]. IL-13 produced in response to murine intestinal parasitic infections stimulated goblet cell hyperplasia and increased Muc2 and Muc3 mRNA levels [78,79]. The dose dependent effect of some cytokines on the changes of mucins have also been reported: *in vitro* experiments on HT29-Cl.16E colonic cells indicated a dose-dependent release of mucin in response to IL-1 [56]. In addition, IFN-γ increased mucin synthesis (measured by radioactive labeled secreted material) in HT29 and LIM-6 intestinal cell lines in a dose-dependent manner, but had no effect on LS174T and Caco2 cell [80].

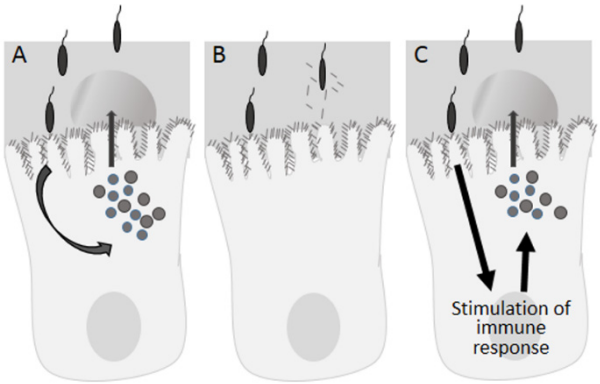

Figure 2. Role of mucin in defense against GI pathogens. Invasion of epithelial surface by enteric pathogens can result in: A) discharge of mucin in direct response to pathogen, B) shedding of cell-surface mucins and clearance of the cell surface from bacteria, C) activation of immune responses that stimulates mucin release.

Mucins and pathogenic invasion

The direct involvement of mucins in preventing pathogenic invasion has been proven by the outcome of studies on mice with a deficiency in secreted or cell-surface mucins. Infection with *Citrobacter rodentium* (*C. rodentium,* an infectious agent

providing a self-limiting mouse model for attaching and effacing *Escherichia coli*) in Muc2 deficient mice ($Muc2^{-/-}$) developed severe, life-threatening disease [81]. The $Muc2^{-/-}$ mice also demonstrated a delay in clearance of the nematode parasite *Trichuris muris* [82]. Furthermore, mice deficient in Muc1 are more susceptible to infection with the gastric pathogen *Helicobacter pylori* (*H. pylori*) [66,83] and the intestinal pathogen *Campylobacter jejuni* (*C. jejuni*) [84]. Despite the general acknowledgment of the importance of mucins in the defense against pathogens, the detailed mechanism of this defense is not fully understood.

Enteric pathogens have developed a range of strategies to subvert and avoid the mucosal layers and reach the underlying epithelial surface, where they can initiate disease. Flagella are a common feature that appears to assist bacterial penetration into the viscous mucus layer [85-89]. In addition, different mechanisms are used by pathogens to alter the mucus layer to facilitate their motility. Some pathogens increase the pH of their surrounding environment to decrease the mucus viscoelasticity [90], while others disassemble the oligomerized mucin macromolecules by proteolytic cleavage and dissolving the mucus gel [91]. In turn, the host response to pathogens includes changes in mucin production, as well as modification of the constituents and biophysical properties of mucus [45]. For example, based on immunohistological staining, infection with *C. rodentium* (in mouse), *Salmonella St Paul, C. jejuni* and *Clostridium difficile* (in human) increased the quantity of Muc1/MUC1 in the colon [36]. Furthermore, long term infection with *H. pylori* changed the mucin glycoslation with a decrease in fucosylation and increase in sialylation [92]. A massive discharge of mucin in response to microbial products has been detected in a range of studies [8,45] (Figure 2A). For example, *in vitro* treatment of cultured intestinal goblet cells with LPS from *Escherichia coli* (*E. coli*), demonstrated up-regulation of MUC5AC and MUC5B mRNA in these cells [93]. In addition, LPS from *Pseudomonas aeruginosa* resulted in an increase in gene expression of MUC5AC and PAS/Alcian blue staining in mouse tracheal epithelial cells [94]. An increase in the level of MUC5AC mRNA in mucoepidermoid cells of the lung has also been detected in response to *Pseudomonas aeruginosa* flagellin in parallel to interleukin-8 (IL-8) secretion [95]. Addition of culture supernatant from *Fusobacterium nucleatum* to human bronchial epithelial cells up-regulated MUC5AC mRNA [96].

The pathogens that do penetrate the inner mucus layer are likely to interact with cell-surface mucins, which restrict their binding to other glycoproteins and neutralize these pathogens. Subsequent to this binding, the cell-surface mucin could shed and act as a decoy to limit the adhesion of pathogens to the epithelial cells [83] (Figure 2B). In addition, there is emerging evidence that indicates adhesion of pathogens to cell surface mucins stimulates the inflammatory signaling cascade of epithelial cells [97] (Figure 2C).

Helicobacter pylori

H. pylori is a micro-aerobic, Gram-negative, helical rod-shaped bacterium that colonizes the gastric mucosa of approximately half of the world's population [98]. Although most people infected with *H. pylori* are asymptomatic, this bacterium has been recognized as the primary cause of several gastric diseases, including chronic gastritis, peptic ulcer disease and gastric mucosa lymphoid tissue lymphoma, and also as the main risk factor for gastric cancer [99-102]. The clinical outcomes of *H. pylori* infections are determined by the host–pathogen interactions. Parameters such as the bacterial genotype, environmental determinants and host genetics are important for the progression of disease [103]. *H. pylori* colonization usually occurs during childhood, and once established the bacteria can remain in the gastric mucosa for life [104]. *H. pylori* isolates have diverse genome sequence and pathogenicity, and different factors are involved in their pathogenesis. The virulence factors such as urease, flagella, adhesins, and toxin delivery systems seem to be crucial for *H. pylori* colonization and establishment of infection in the gastric mucosa. To facilitate the colonization of the gastric mucosa, the urease enzyme of *H. pylori* converts urea to ammonia ions, and elevates the pH of the microenvironment around the bacteria, which protects it from the acidic gastric lumen [105,106]. The increase in pH also facilitates bacterial penetration into the mucus layer by transforming the viscoelastic mucus gel (at low pH), that effectively traps the bacteria, to a viscoelastic solution [107]. In the suitable environment provided by urease, flagella mediate the motility of *H. pylori;* it may enable the bacteria to penetrate the mucus gel and to remain near the epithelial layer despite the rapid turnover of mucus layers. The movement of *H. pylori* is dependent on a unipolar bundle of two to six sheathing flagella, and this sheathing is also believed to protect the bacteria from the acidic environment in the stomach [108]. An additional important factor in the motility of *H. pylori* is its helical rod shape, which may enable the bacterium to swim faster in the mucus layer [109]. After penetration, *H. pylori* persists in the stomach, possibly by using several outer membrane proteins, which adhere to the corresponding receptors on the host gastric epithelium. A number of adhesins have been identified on *H. pylori* such as surface-located heat shock protein [110], HopZ [111], AlpA/AlpB [112] and OipA [113]. However, the most studied adhesins are the blood group antigen binding adhesin (BabA), sialic acid binding adhesin (SabA), cytotoxin-associated gene L (CagL) that respectively binds to fucosylated structures such as lewis b antigen (Le^b), sialyl-Le^x and integrin [114-117]. BabA can also act as a mediator for *H. pylori* binding to MUC5AC, even in non-secretors or those without Le^b [118]. Following colonization, *H. pylori* delivers toxins into the epithelium, mediated through the vacuolating cytotoxin (VacA) and cytotoxin-associated gene A (CagA). The VacA toxin has pore-forming activity and induces vacuolation and apoptosis in epithelial cells [119], as well as inhibition of T lymphocyte proliferation [120,121]. CagA is a

member of the cytotoxin-associated gene pathogenicity island, which expresses a type IV secretion system and induces a pro-inflammatory response and multiple cytoskeletal and gene regulatory effects on gastric epithelial cells [122].

H. pylori invasion can be recognized by a variety of putative pathogen recognition molecules in the host, which are capable of regulating the innate and adaptive immune responses through recognition of conserved microbial components. The result is the production of inflammatory cytokines such as interleukin-8 (IL -8), interleukin-12 (IL-12), IL-6, IL-1β, interleukin-18 (IL-18), interleukin-10 (IL-10), and TNF-α [123-125]. Long term colonization of the gastric mucosa by *H. pylori* induces the adaptive immune system to elicit Th1 and Th17 cell responses, which results in elevated levels of the cytokines IFN-γ and IL-17 [126,127]. In general, despite the effort of the host immune system to eradicate *H. pylori* from the mucosal surface, this bacterium has evolved defense mechanisms which secure its long term colonization.

Gastric mucin and *H. pylori*

Several studies have demonstrated that *H. pylori* bind to the MUC5AC and MUC1 mucins [128-130]. Furthermore, MUC1 can block the binding of *H. pylori* to the epithelial surface, though the mechanisms are different between strains with BabA and SabA and the strains without these adhesins. Infection of cultured gastric cells with *H. pylori* strains expressing these adhesins causes the shedding of MUC1 from the cell surface, coats the bacterium and acts as a decoy to limit binding to the epithelial layer [83]. Conversely, in the strains which lack BabA and SabA, the mucin blocks binding of the bacterium to the cell surface by steric hindrance [83]. Mucins can also influence the proliferation, gene expression, and virulence of *H. pylori* [131].

In addition, there appear to be changes of the mucin expression during infection based on the outcome of several studies, although there is some controversy about the decrease of MUC5AC levels. The immunohistochemical studies on human biopsy specimens from *H. pylori* infected individuals detected no difference in MUC5AC protein levels [132-134], while another study demonstrated a reduction of both MUC5AC and MUC5AC expressing cells [135]. Furthermore, an *in vitro* study using the gastric cancer cell line KATO-II infected with *H. pylori* found that mucin synthesis decreased upon infection [136,137]. MUC6, which is normally produced by gastric gland mucous cells, have been reported to be expressed in surface mucous cells in *H. pylori* infected patients [134]. In human biopsy samples the thickness of the adherent mucus layer remained intact in infected patients [138], whereas *H. pylori* infection in mice decreased the thickness of the adherent mucus layer [139].

Infection with *H. pylori* can also lead to intestinal metaplasia, with the stomach mucus developing characteristics of intestinal mucus [140]. MUC6, which is normally produced by gastric gland mucous cells, have been reported to be expressed in surface mucous cells in *H. pylori* infected patients [134]. MUC6 from one patient could inhibit proliferation of *H. pylori* in a dose-dependent manner, and thereby act as a natural antibiotic, potentially decreasing the bacterial burden [141].

H. pylori SS1 (Sydney strain)

To achieve a better understanding of the *in vivo* mechanism of the *H. pylori* infection, many investigators have chosen to use the mouse as a model. However, most of the strains obtained from patients have low colonization kinetics and a weak phenotype in mice. In 1997, Lee and colleagues in Australia successfully adapted the Sydney strain-1 (SS1) isolates of *H. pylori* through serial inoculation in the mouse stomach [142]. Today, the *H. pylori* SS1 strain is the most commonly used infecting agent for mouse studies, that colonizes, in particular, the C57BL/6 strain for longer periods and with a higher number of bacteria compared to other available strains of mice [142]. *H. pylori* SS1 can colonize and produce gastritis in the mouse, although the infection with this strain does not exactly mimic human gastritis. Moreover, the formation of gastric ulcers and cancer has not been observed in mice during infection with the SS1 strain [143]. However, the long-term pathology has more in common with human chronic gastritis in comparison to other strains of helicobacter [142].

Escherichia coli

E. coli a gram-negative, rod-shaped bacterium, is an incredibly adaptable and diverse enterobacterial species. *E. coli* can be subdivided into the intestinal non-pathogenic (commensal isolates), intestinal pathogenic isolates and extraintestinal pathogenic *E. coli* or ExPEC isolates, based on genome content and phenotypic traits. Several intestinal pathogenic subspecies of diarrheagenic *E. coli* have been described: enteroinvasive *E. coli*(EIEC), cnteroadherent or enteroaggregative (EAEC), enterotoxigenic (ETEC), enteropathogenic (EPEC) and enterohaemorrhagic (EHEC), based on the clinical syndromes they can cause and their virulence traits [144].

ETEC

Enterotoxigenic *Escherichia coli* (ETEC) is one of the six recognized diarrheagenic *E. coli* and the most common cause of diarrhea in children in developing countries, and also in travelers in those areas. Approximately, 200 million diarrheal episodes

and 380,000 deaths are caused by ETEC annually [145]. The pathogenicity of ETEC depends on its colonization and virulence factors. Adherence of ETEC to the intestinal mucosa is facilitated by colonization factors (CFs) that are remarkably selective for host and tissue. CFA/I and CS1 to CS6 are generally more prevalent than other identified CFs in human ETEC [146]. Following colonization, the virulence factors of ETEC, which consists of heat-labile enterotoxin (LT) and/or heat-stable enterotoxin (ST), stimulates net secretion of ions and water, causing watery diarrhea [147]. In addition to these virulence factors, ETEC has evolved other mechanisms to overcome the protective effect of the mucus layer to invade the epithelial surface. Recent *in vitro* studies demonstrated the ability of ETEC in degrading MUC2, which accelerated toxin access to the enterocyte surface [148,149]. In addition, previous studies in pig established binding of the cell-surface mucins MUC4, MUC13 and MUC20 to the CFs of porcine ETEC [150-152], which can block the binding of CFs to the epithelial surface. An *in vitro* experiment with porcine intestinal cells demonstrated downregulation of expression of MUC4 and MUC13 by the colonization factors fimbrial types (F4ab and F4ac respectively) of porcine ETEC [153]. In addition, knockdown of MUC13 in the same epithelial cells resulted in an increase of ETEC adhesion to the cells [154]. The other aspects of immune response to ETEC are mainly mediated through secretory IgA directed towards the LT toxin and colonization factors [155]. Stimulation of human T cells with LT and CFs results in a Th1/Th17 response [156,157] while intranasal immunization in mice was reported to effect a mixed response more directed towards a Th2 response [158].

Attaching and effacing pathogens

Enteropathogenic *Escherichia coli* (EPEC) and Enterohemorrhagic *E. coli* (EHEC) are members of the attaching and effacing (A/E) family of pathogens that induce histopathological lesions, termed A/E lesions, on the apical surface of the host enterocytes [159]. The A/E lesions are characterized by localized destruction of brush-border microvilli and intimate attachment of the bacteria to the plasma membrane of the host epithelial cells [160]. EPEC is a cause of gastroenteritis in infants while EHEC causes bloody diarrhea in children and the elderly. These pathogens are distinct from each other based on production of Shiga toxins by EHEC, that cause kidney damage leading to the hemolytic uremic syndrome, a form of acute renal failure [161]. The A/E pathogens carry a pathogenicity island, the locus of enterocyte effacement (LEE) that encodes gene regulators, the adhesin called intimin, a type III secretion system, chaperones, and several secreted proteins, including the translocated intimin receptor (TIR) [162]. A variety of effectors are shared by all A/E pathogens [163], which subvert different host cell processes, and

enable the bacteria to colonize, multiply and cause the disease. The type III secretion system which is an injection device (injectisome), transfer bacterial virulence proteins directly into host cells [164] using two distinct categories of proteins: the translocators that form a pore in the target membrane and the effectors that transport through this pore into the host cell [162]. Therefore, the EHEC/EPEC remains mostly extracellular in the lumen of the intestine, while T3SS injected effectors access and manipulate the intracellular environment of the host cells. The effectors can regulate the host cellular functions involved in the immune response, the cytoskeleton dynamics and the maintenance of tight junctions.

Citrobacter rodentium

C. rodentium is a gram-negative, anaerobic rod which produces transmissible murine colonic hyperplasia, characterized by epithelial cell hyperproliferation in the descending colon [165]. Infection with *C. rodentium* in most adult mice is subclinical and self-limiting, resulting in slight morbidity or mortality [166]. Oral transmission of this bacteria in mice initiates with the passage through the cecum, followed by colonization of the colonic epithelium, by formation of A/E lesions [167] equivalent to that caused by EPEC and EHEC in human. *C. rodentium* also possesses a homologue of the LEE pathogenicity island as described in EPEC/EHEC. These similar traits make *C. rodentium* a useful model for *in vivo* studies of the molecular basis of LEE-mediated pathogenesis, and the mechanisms underlying mucosal responses to infection, especially since EPEC and EHEC do not infect adult mice efficiently [168].

Host defense against A/E pathogens

A key step for A/E pathogen to invade the epithelial surface appears to be to overcome the barrier produced by the outer and inner mucus layer. Indeed, *C. rodentium* infection of $Muc2^{-/-}$ mice indicated extreme susceptibility to infection, induced mortality and disease, as well as faster colonization and higher pathogen burdens throughout the course of infection in mice lacking a mucus layer [81]. The protective effect of the mucus indicates that the bacterial factors involved in crossing this layer are presumably critical for virulence. As *C. rodentium* is non-motile due to the lack of a functional flagellum, the ability of the bacteria to overcome the mucus barrier has been speculated to utilize specific mucinases or glycosidases that digest mucins [169]. The fact that EHEC has been shown to secrete the metalloprotease StcE with apparent mucinase activity [170] suggested that A/E pathogens could employ this strategy. Furthermore, in *C. rodentium* infected WT mice an increase in luminal mucus was detected at day six post infection compared to non-infected mice, which could indicate that Muc2 promotes host defense by flushing *C. rodentium*

away from the mucosal surface and out of the colon [81]. In addition to the mucus layer, the other components of the host immune response that have been shown to be necessary for pathogen clearance, are $CD4^+$ T cells, B cells, mast cells, and neutrophils [171-175]. IgG responses are also required, whereas IgA and IgM responses are not essential [172]. Infection with A/E pathogens leads to elevated Th1 and Th17 responses, and mice lacking the cytokines needed for these responses (IFN-γ, IL-12, IL-17, and IL-22) have increased susceptibility to *C. rodentium* [176-178]. It was also determined that the IFN-γ produced by $CD4^+$ T cells is essential for controlling pathology and bacterial density [179], although mice with a deficiency in IFN-γ cytokines are still capable of clearing *C. rodentium* infection, albeit with a longer duration of infection [176].

AIMS OF THE PROJECT

General aim

The overall aim of this thesis was to understand the effect of bacterial infection on mucin production and turnover in the gastrointestinal tract.

Specific aims:

I. To evaluate the production rate and turnover of mucins in the murine gastric mucosa in non-infected mice, and mice with early colonization and chronic *H. pylori* infection.

II. To develop an *in vitro* model that resembles the mucosal surface of the gastrointestinal tract by producing polarized cells, functional tight junctions, and a mucus layer.

III. To investigate the mucus and mucin dynamics, as well as goblet cell and enterocyte function during infection and clearance in the self-limiting *C. rodentium* infection model.

IV. To define the effect of cytokine environment on changes in the intestinal mucus layer during infection with *C. rodentium* and ETEC.

METHODOLOGICAL CONSIDERATIONS

The methods used in this thesis are described in the attached papers and manuscript. Only more detail on the method for preserving the intestinal *in vitro* model is described here.

Carnoy's fixation and preservation of *in vitro* mucosal surfaces (Paper II and IV)

Previous work on establishing fixation procedures for preserving an intact mucus layer demonstrated the importance of avoiding water in the fixatives [134,180]. Carnoy's fixative with dried methanol as the alcohol component (methanol Carnoy) was found to be the most efficient preservative for the mucus. Therefore, in our experiments, this fixative was used to preserve the *in vitro* mucosal surface. To minimize damage, the membranes were fixed and paraffinized without removing the inserts. To keep the secreted mucus intact two methods were used. One method was by making a "sandwich" in which a second membrane that had undergone identical treatment was cut out of the insert and flipped on top of the other membrane in a manner where the apical surfaces were facing each other. The other method used was to cover the apical surface of the *in vitro* membrane with a thin layer of bovine liver, followed by immediate soaking in the methanol Carnoy fixative to reduce the possible effect of enzymes from liver on the mucus layer.

RESULTS

Maintenance of gastrointestinal mucosal homeostasis is dependent on the balanced and dynamic interactions between mucus layer, intestinal epithelial cells, microbiota, and the host immune defense [181-183]. To establish infection, the enteric pathogens have to disrupt the gastrointestinal homeostasis and cause a defect in the mucus barrier to increase permeability. Penetration of pathogens into the mucus layer leads to inflammation and disruption of the gastrointestinal epithelial cells. Currently, the focus of many studies is on the understanding of the crosstalk between host mucosal defense and pathogens. One of the obstacles in understanding the mechanisms of these interactions is that many pathogens are human specific and the animal models, although useful, have disadvantages when it comes to relating the results to human disease. Therefore, to determine the mechanisms of infection, *in vitro* models are necessary. However, the common *in vitro* cell cultures are suboptimal, as they are often not polarized, lack important components of the glycocalyx, and have very low production of secreted mucin. Hence, the first objective of this thesis was to establish a cell culture method to create reproducible *in vitro* mucosal surfaces that better mimic the *in vivo* mucosal surface of the gastrointestinal tract. My thesis was originally intended to focus on *H. pylori* infection. However, despite our effort, none of the gastric cell lines tested formed an enhanced mucus layer in response to the range of applied culture methods used. Due to the combined lack of a suitable gastric *in vitro* model, technical problems with measuring electrical parameters of the murine stomach as well as with preserving the gastric mucus layer intact, we decided to also include the intestinal pathogens *C. rodentium* and ETEC.

In vitro mucosal surfaces suitable for infection studies, created by Semi-wet interface culture (Paper II)

The major traits for creating *in vitro* mucosal surfaces suitable for infection studies, are the abilities to produce continuous adherent polarized layers with functional tight junctions, and a mucus layer. To create this layer, we started with the previously used air-liquid interface culture condition that change the airway and rabbit conjunctival epithelial cells into polarized cells [184,185], and made a series of modification experiments leading to a method of semi-wet interface cultivation with mechanical stimulation that provides an environment resembling the gastrointestinal milieu. The response of different cell lines to this treatment was diverse, and some of them were capable of forming firmly adherent continuous polarized layers, whereas other cell lines did not have this ability. Amongst tested intestinal cell lines, Caco-2, T84, LS513 and HT29 MTX (-P8 and –E12) were capable of producing adherent epithelial layers (Caco-2 and T84 do this also in the absence of this treatment).

MKN7 cells were the only gastric cell line that produced an adherent polarized epithelial layer in response to the semi-wet interface. The mechanical stimulation had a negative effect on the integrity of these cells. The cell lines also varied in mucin expression, and none of them produced more than 1% of the amount of the mucins that builds up the mucus layer in the *in vivo* gastrointestinal mucosa [186]. The mechanical stimulation and continuous wetting of the apical surface used in our culture method, provided a more homogenous surface, stimulated mucus production and altered the morphology of the LS513, Caco-2, T84 and especially HT29 MTX (-P8 and –E12) intestinal cells to a three-dimensional structure with some very shallow crypts. The addition of a Notch γ-secretase inhibitor (DAPT), which is known to promote goblet cell differentiation [187], as a chemical stimulator enhanced the thickness of the mucus layer. In summary, the culture of intestinal cell lines LS513, and HT29 MTX-P8 and HT29 MTX-E12 in a semi-wet interface with mechanical and chemical stimulation resulted in production of an *in vitro* mucosal layer with considerable resemblance to the *in vivo* environment. The polarized epithelial surfaces covered with a relatively thick mucus layer produced from the HT29 MTX (-P8 and –E12) cells, is similar to the environment of the *in vivo* mucosal surface during interaction with pathogens. In addition, the reproducibility of these *in vitro* models, especially HT29 MTX-E12 cells, made them suitable model candidates for *in vitro* infection studies. Therefore, we chose this cell line for our infection studies, and from here on we refer to the HT29 MTX-E12 culture in semi-wet interface with mechanical and chemical stimulation as *in vitro* mucosal surfaces.

Changes in mucus layer, mucins and goblet cells in response to infection and cytokines (Paper III and IV)

Previous studies in the gastrointestinal tract indicated that bacterial infection either induces goblet cells and mucin synthesis with frequent secretion, or depletes the goblet cells followed by quantitative and qualitative alteration in the mucus layers [182,188]. These studies usually focus on a single time point, and the regulatory cascade involved in the response mechanism of mucin producing cells to the pathogens is poorly defined. The current knowledge envisions two possible pathways: direct effects of microbes on goblet cell functions through the local release of the microbial components; or changes in goblet cell function in response to host-derived bioactive factors generated by activated epithelial or underlying immune cells after interaction with pathogens [189]. To gain a better understanding of the effect of pathogens and the host immune system on changes of the mucus layer, we used the self-limiting mouse pathogen *C. rodentium* infection as a model for A/E pathogens. We examined the variation in the number of goblet cell, the amount of stored mucin and the thickness of the mucus layer of WT mice (C57BL/6) during

different phases of infection, from early infection to the clearance of the pathogen (paper III). The results showed a progressive change that already began at early colonization (4 days post infection). At this time point the thickness of the adherent mucus layer decreased to the lowest level, and there was goblet cell depletion, but the engorgement of the mucins inside the goblet cells increased to its highest level. During day 10 post infection when the fecal *C. rodentium* reached the highest density, mucus thickness remained as low as day 4 post infection, followed by substantial goblet cell depletion with a reduction of the total amount of mucin inside the goblet cells. In addition, at this time point, the organized adherent inner mucus layer present in uninfected mice was not found in the infected mice. From day 14 post infection, when the bacterial density reduced to day 19 which is when there was the clearance of infection, there was a continuous increase in the number of goblet cells, and the mucus layer reached the greatest thickness, which was even more than non-infected animals. At day 14 the mucin engorgement decreased to its lower level, which could be based on the release of mucins into the mucus layer. Figure 3 summarizes the changes during the time course of infection.

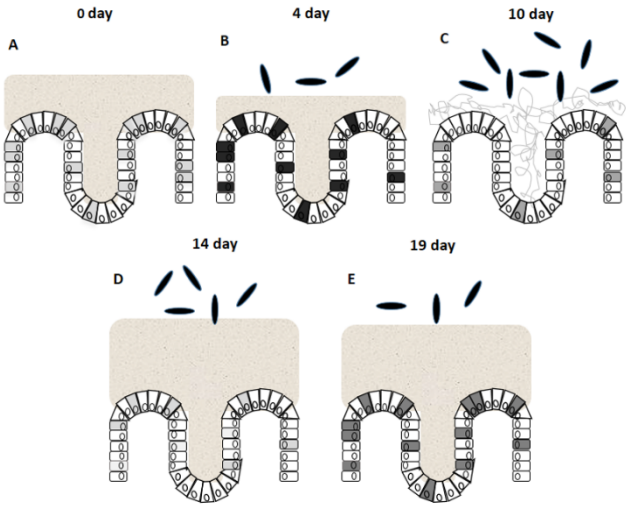

Figure 3. Schematics of changes in goblet cells during *C. rodentium* infection in WT mice. The total amount of mucin inside the goblet cells shown in different shades of gray to indicate the intensity of stored mucins. Black is used for the highest amount of mucin inside the goblet cells. The thickness of the mucus layer is shown as a gray surface for the organized adherent inner mucus layer, and curled lines indicate an unorganized layer.

To examine the effect of the cytokine environment on the changes of mucins and mucus layer, we compared differences in mucin synthesis and the mucus layer thickness between wild-type (WT) and interferon deficient mice (IFN-$\gamma^{-/-}$), since previous data demonstrated the importance of the Th1 response, especially the IFN-γ cytokine in protection against *C. rodentium* infection [176,190]. The outcome showed that at the time point of infection with the highest bacterial load, IFN-$\gamma^{-/-}$ mice had a thicker mucus layer, more engorgement of mucins and less goblet cell depletion compared to the WT mice (paper IV).

We then examined the effect of cytokines involved in the Th1/Th2 response, individually or as a combination with and without *C. rodentium*/ETEC infection, on our recently developed *in vitro* mucosal surfaces. Selections of cytokines were based on the differences in their expression level in WT versus IFN-$\gamma^{-/-}$ mice at day 10 post infection, and/or the gene upregulation during the clearance phase, when the mucus layer is enhanced (Table1).

***In vivo* infection**	IFN-γ	TNF-α	IL-12	IL-6	IL-4	IL-13
mRNA level 10 d p.i in IFN-$\gamma^{-/-}$ and WT mice	↓	-	↓	↑	↑	↑
mRNA level of WT mice during clearance (day 14 and19 post infection)	↑	↑	↑	↑	↑	↓

Table 1. Changes in the mRNA level of cytokines involved in Th1/Th2 response during *C. rodentium* infection.

A summary of the outcome of the study is shown in Table 2. In the absence of infection, treatment with the Th1 cytokines (IFN-γ, TNF-α) and their combination reduced the number of goblet cells and the morphology score of the membranes, which is an indicator of mucin engorgement in the surface goblet cells. In contrast, IL-4 and IL-13, which are cytokines involved in the Th2 response, increased the number of goblet cells and enhanced the bulkiness of the surface goblet cells. Infection with *C. rodentium* seemed to reverse the effect of cytokine treatments on the *in vitro* mucosal surfaces, making them more similar to untreated membranes. Infection with ETEC had the same nullifying effect as *C. rodentium* on the membranes treated with IFN-γ and TNF-α, although, they did not reverse the morphological enhancement caused by IL-4 and IL-13. Infection with these pathogens had no effect on the membranes treated with the combination of IFN-γ and

TNF-α with and without the addition of IL-4. In general, these results indicated changes in the goblet cells, mucin and mucus layer during infection is dependent on the combined impact of the pathogen and cytokines. Therefore, these parameters should be considered in parallel during the studies on the interaction between mucin and pathogens.

***In vitro*: Non-infected**	IFN-γ	TNF-α	IFN-γ + TNF-α	IFN-γ + TNF-α + IL-4	IL-12	IL-6	IL-4	IL-13
Effects on Morphology of *in vitro* mucosal surface	↓***	↓***	↓***	↓***	↑	x	↑*	↑
Effects on goblet cell density of *in vitro* mucosal surface	↓	↓**	↓***	↓***	↑	↑**	↑*	No
***In vitro*: *C. rodentium* infection**	IFN-γ	TNF-α	IFN-γ + TNF-α	IFN-γ + TNF-α + IL-4	IL-12	IL-6	IL-4	IL-13
Effects on Morphology compared to the non-treated of *in vitro* mucosal surface	No	No	↓***	↓***	No	No	No	No
Effects on Morphology compared to the same treatment in the non-infected *in vitro* mucosal surface	No	No	No	No	↓***	No	↓*	↓*
Effects on goblet cell density compared to the non-treated *in vitro* mucosal surface	No	No	↓**	↓*	No	No	No	No
Effects on goblet cell density compared to the same treatment in the non-infected *in vitro* mucosal surface	No	No	↓***	↓***	No	No	No	No

***In vitro*: ETEC infection**	IFN-γ	TNF-α	IFN-γ + TNF-α	IFN-γ + TNF-α + IL-4	IL-12	IL-6	IL-4	IL-13
Effects on Morphology compared to the non-treated of *in vitro* mucosal surface	No	No	↓***	↓***	No	No	No	No
Effects on Morphology compared to the same treatment in the non-infected *in vitro* mucosal surface	No	↑***	No	No	No	↓*	No	No
Effects on goblet cell density compared to the non-treated *in vitro* mucosal surface	No	No	↓***	↓***	No	No	No	No
Effects on goblet cell density compared to the same treatment in the non-infected *in vitro* mucosal surface	No	No	↓***	↓**	No	No	No	No

Table 2. Summary of changes in morphology and goblet cell density of *in vitro* mucosal surfaces treated with Th1/Th2 cytokines, with and without *C. rodentium*/ETEC infection. Statistics: One-way ANOVA, Bonferroni post hoc's, *<0.05, **<0.001, ***<0.0001. (n=4-12)

The epithelial response to ion transport secretory stimulators (Paper II, III)

The presumption of the epithelial tissue as an electrical circuit, is the basis for the commonly used Ussing chamber method for measurement of the electrical parameters of epithelial layers. In this method, the membrane current (Im) and transepithelial potential (PD) is used to measure the active transport of ions across the epithelium, epithelial resistance (Rp) to monitor tissue integrity [191-193], and membrane capacitance (Cp) to study exocytosis of mucins of single cells and cultured epithelial monolayers [194-196]. In our project, we used the measurement of the electrical parameters as a baseline to compare the ability of different gastrointestinal cell lines in the production of integrated *in vitro* epithelial surfaces. A

low resistance and a fluctuating PD were related to the inability of the cell lines to produce an adherent monolayer with tight junctions. This was in line with the results of PAS/Alcian blue staining of the membranes that showed unorganized layers of cells. Among the tested cell lines, T84, Caco2, LS513, HT29 MTX-P8 and HT29 MTX-E12 intestinal cells produced a layer with a high resistance (over 100 $\Omega*cm^2$), while MKN7 was the only gastric cell line that provided a resistance (paper II). We also measured the Rp and PD of the mice colonic epithelial layer during *C. rodentium* infection, to compare the electrophysiological changes of this layer during different phases of infection. The outcome showed a substantial reduction of Rp and PD during the most severe colitis at day 10 and 14 post infection, although the magnitude of these parameters correlated weakly with the colitis score. Since we detected the low level of Rp and PD in the *in vitro* surfaces is associated with the lack of membrane integrity, we can speculate that the decrease of these electrical parameters in the infected mice is related to the increase of paracellular permeability. This is also in line with previous studies that demonstrated an increase in paracellular permeability in *C. rodentium* infected mice [197].

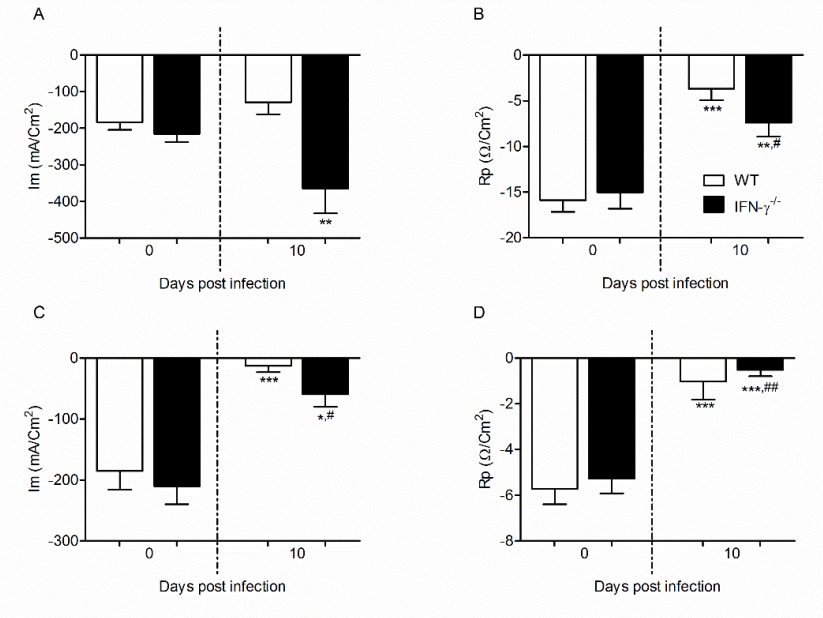

Figure 4. The response to forskolin and charbachol is altered during infection in distal colon explants studied in Ussing chambers. Changes in Im (A and C) and Rp (B and D) in response to forskolin (A-B) and charbachol (C-D) in distal colonic tissue during *C. rodentium* infection of WT and IFN-$\gamma^{-/-}$ mice. Statistics: ANOVA bonferroni's post hoc test, *p<0.05, **p<0.001 and ***p<0.0001vs day 0 of the WT mice, # p<0.05 and ## p<0.001 vs day 0 of IFN-$\gamma^{-/-}$ mice. (n=8-17)

We also measured the electrical parameters of the *in vitro* mucosal surfaces to study the ability of different cell lines in response to the secretagogues such as carbachol (CCh) and forskolin, which are involved in activation of the ion secretion pathway. The existing data on CCh demonstrated its effect on induction of the mucus secretion in the small and large intestine [198,199]. *In vitro* experiments demonstrated that stimulation with CCh is mediated via the Ca^{2+} pathway of ion secretion, and only induced secretory responses in LS513 cells, which is a goblet cell-like cell line, and had no effect on enterocyte-like cell line Caco-2 (paper III). We also detected mucus release from the other goblet cell-like cell lines (HT29 MTX-P8 and HT29 MTX-E12) into the mucus layer, in response to addition of CCh to the basolateral side of the membranes. This effect was detected by an increase in the epithelial capacitance, and PAS/Alcian blue staining of the membranes (paper II). In addition, both goblet cell-like and enterocyte-like cell lines demonstrated changes in Im and Rp, which reflected ion channel activity in the epithelium, in response to stimulation with forskolin, which mediates the cAMP pathway of ion secretion. The ability of *in vitro* mucosal surfaces in responding to these stimulators is important for the infection studies, as evidence showed that during infection and inflammation, enteric pathogens induced mucus and fluid secretion, which coincides with ion secretion. The ion secretion may contribute to mucus hydration and clearance of pathogens, although the molecular mechanism of the process and the relative importance of chloride and bicarbonate secreted by these ion channels, in this pathway is not fully understood. However, recent experiments with mice ileum showed the importance of bicarbonate for the formation of a normal mucus layer, as it could expand the stored Muc2 mucin at secretion [29].

In our *in vivo* studies, there was a gradual decrease in the Rp of the colonic explant of *C. rodentium* infected WT mice in response to both forskolin and CCh from day 0 to day 14 post infection which was restored to the pre-infection level after clearance (day 19 post infection). However, only CCh could change the Im and forskolin had no effect (paper III). In contrast, the Im of IFN-$\gamma^{-/-}$ mice had a noticeable increase in response to forskolin stimulation at day 10 post infection (Figure 4A). Stimulation with either CCh or foskolin, however, had no effect on changes in Im and Rp of the non-infected WT and IFN-$\gamma^{-/-}$ (Figure 4A-D). This could indicate higher activity of ion channels in IFN-$\gamma^{-/-}$ mice during infection, which coincides with the increase of mucus thickness in these mice at the same time point.

In summary, these results demonstrate that the electrochemical parameters and the ion transport of the epithelial layer changes during infection, and this variation also extended to the changes in response to different stimuli. Interestingly, the increase of mucus thickness in both WT and IFN-$\gamma^{-/-}$ mice coincide with the altered ion channel activities.

Mucin production rate and turnover in the gastrointestinal infection (Paper I and IV)

Penetration of pathogens into the protective mucus barrier could result in modification of the structure and synthesis of mucins that are the main component of the mucus layer. The changes in mucin synthesis and secretion could be regulated by pathogens and/or components of the immune response [45]. However, there is a gap of knowledge about the effect of different pathogens and cytokines on mucin synthesis during infection. In this work, using labeling of the *O*-linked mucin type glycans, we investigated the effect of gastrointestinal pathogens as well as cytokines involved in the response to these pathogens, on the biosynthesis of mucins. The method is applicable both *in vitro* and *in vivo* and is based on incorporation of the GalNAc analogue GalNAz into the mucins during biosynthesis. The visualization occurs using click-it chemistry that attaches a fluorescent alkyne to the incorporated GalNAz [200,201]. Previous results from the murine distal colon reported a duration of 6 to 8 h from incorporation of GalNAz to release of the labeled mucin to the lumen [202]. Therefore, we performed a 12 h study to obtain the basal production rate and turnover of mucin in both murine stomach and the intestinal *in vitro* mucosal surfaces. The metabolic incorporation of GalNAz into the newly synthesized glycoproteins was detected from the first hour after injection/addition both *in vivo* and *in vitro*. The newly synthesized mucins were localized at the supra nuclear part of the surface epithelial cells/goblet cells after 1 h of GalNAz incorporation. During the second and third hours, the mucins moved through the cells toward the cell surface. Five hours after injection, a slight difference was detected between the two systems. In the surface cells of the murine stomach, some newly synthesized mucins were detected on the cell surface, whereas some remained inside the cell, while, in the *in vitro* mucosal surfaces, some of the goblet cells have released their GalNAz labeled mucin into the mucus layer. The traces of the newly synthesized mucins in the murine gastric mucus layer were detected after 6 h of injection, although in both systems, the mucins could still be detected inside the majority of the cells. During the time points of 6 to 11 h post addition of GalNAz into the *in vitro* model, the proportion of cells having released their newly synthesized mucins into the mucus layer increased with time, and after 12 h most of the goblet cells had released their GalNAz labeled mucins to the lumen. The same trend was detected in the murine stomach, although even after 12 h some of the surface epithelial cells still had not released their newly synthesized mucins. Based on these results, we chose the major transitional stages of the mucin turnover: the transfer to the cytoplasm, the start point of the release of the mucin to the mucus layer, and the time point that most of the cells have released their newly synthesized mucins into the mucus layer, to study the effect of different cytokines and pathogenic infection on mucin turnover. Therefore, the time points for *in vitro* study were slightly different from the *in vivo* experiments.

***In vitro*: Non-infected**	IFN-γ	TNF-α	IL-12	IL-6	IL-4	IL-13
Effects on turnover of *in vitro* mucosal surface	↓	↓	↓	↑	↑	↑
***In vitro*: *C. rodentium* infection**	IFN-γ	TNF-α	IL-12	IL-6	IL-4	IL-13
Effects on turnover compared to the non-treated *in vitro* mucosal surface	↑	↓	↑	↑	↑	↑
Effects on turnover compared to same treatment in the non-infected *in vitro* mucosal surface	↑	↓	↑	↑	↑	↑
***In vitro*: ETEC infection**	IFN-γ	TNF-α	IL-12	IL-6	IL-4	IL-13
Effects on turnover compared to the non-treated *in vitro* mucosal surface	↑	↑	↑	↑	↑	↑
Effects on turnover compared to the same treatment in the non-infected *in vitro* mucosal surface	↑	↑	↑	↑	↑	↑

Table 3. Mucin turnover in intestinal *in vitro* mucosal surfaces treated with Th1/Th2 cytokines with and without *C. rodentium*/ETEC infection.

The outcome of the pulse study in the antrum of the *H. pylori* infected mice demonstrated a reduction in rate and turnover of mucin compared to the non-infected mice (paper I). The effect was more prominent in the early colonization than in chronic infection. The results also showed a slower production rate and transfer of the GalNAz labeled mucin in the corpus compared to the antrum of non-infected mice, and this difference was more pronounced during infection (paper I). No difference was detected in the rate and turnover of mucin synthesis in the corpus of non-infected mice and mice with early colonization (paper I). In contrast to the inhibitory effect of *H. pylori in vivo*, infection of the intestinal *in vitro* mucosal surfaces with either *C. rodentium* or ETEC increased the rate and turnover of the newly synthesized mucin, and ETEC had a more accelerating effect (paper IV). The changes in mucin rate and turnover were also detectable in the cells treated with different cytokines involved in the Th1/Th2 response (Table 1 and paper IV). In the absence of infection, the Th1 cytokines (IFN-γ, TNF-α and IL-12) reduced the incorporation of GalNAz and turnover of the newly synthesized mucins, whereas

Th2 cytokines (IL-4, IL-13 and IL-6) had a stimulatory effect (Table 3 and paper IV). In summary, these results indicated that the turnover of mucin is tissue specific, and the cytokine environment as well as pathogens have an important role in the regulation of mucin synthesis and turnover. Indeed, infection of the intestinal *in vitro* mucosal surface with *C. rodentium* and ETEC demonstrated that bacterial infection might overcome the effect of cytokines on goblet cells.

DISCUSSION

In vitro mucosal surfaces – a model for infection studies

In the present thesis, the aim was to study the effect of bacterial infection on the mucus layer and mucins as the main component of the defensive barrier of the gastrointestinal tract. First, we developed a method of cell culture to produce an *in vivo* like *in vitro* mucosal surfaces from intestinal cell lines and used it in parallel to the *in vivo* infection studies in mice. A suitable *in vitro* model is required as most of the enteric pathogens, including the ones that are the main focus of this thesis, are host specific. Therefore, the studies on animal models are not optimal for understanding the interaction between host and pathogens during colonization and infection. In addition, the standard methods of cell culture that are used in many studies cannot produce a polarized epithelial layer, which is an important factor in bacterial adhesion and invasion as well as producing a host immune response to the invading pathogens [203,204]. Furthermore, the common cell lines used for the *in vitro* infection studies have a highly variable expression of mucins and have very low production of gel forming-mucins [186]. Our semi-wet interface culture with mechanical and chemical stimulation produced an adherent polarized epithelial layer, with functional tight junction and relatively thick mucus layer from HT29 MTX (-P8 and -E12) intestinal cell lines. The results of immunohistochemical studies indicated that HT29 MTX (-P8 and -E12) among the tested cell lines, had the greatest resemblance to the human colon mucin profile. In addition, infection of the *in vitro* mucosal surfaces with different pathogens, especially *Citrobacter jejuni*, located the majority of bacteria in the mucus layer in close contact to MUC2. The binding is important in preventing the invasion of the epithelial surface by pathogens and was also detected during *Citrobacter jejuni* infection in chicken and human [45,205]. Furthermore, since massive discharge of mucins from mucin producing cells in response to microbial products or components of immune response is an important aspect in infection studies [49-58], the ability of the *in vitro* model in releasing mucins in response to stimuli such as CCh [46], increases the similarity of this *in vitro* model to the *in vivo* environment. In summary, the methods developed herein, create *in vitro* mucosal surfaces suitable for host-pathogen interaction studies at the mucosal surface.

Effect of infection and cytokine environment on mucins and mucus layer

In the studies for this thesis, we investigated the distinct aspects of the mucus layer and mucins, to gain a better understanding of the effect of infection and the cytokine environment on mucin production and synthesis. Our main focus was the *C. rodentium* infection as a model for A/E pathogens. We investigated the effect of infection on changes in the number of goblet cells, the amount of stored mucin inside the goblet cells, thickness of the adherent mucus layer, mRNA level of different mucins, the electrophysiological properties of the colonic epithelia, and gene expression of cytokines involved in the Th1/Th2 response during the time course of infection. The difference between our work and other studies in this field is that usually in the infection experiments, the focus is only on one aspect of mucin synthesis. Most of the results are based on the mRNA level which is the basal level of mucin synthesis [50,76,78,79,132,133]. However, the mature mucin which is released from the cell into the cell surface or mucus layer is subjected to a great deal of post-translational modification, especially glycosylation, which plays an important role in type, rate and turnover of mucin production [15]. In our studies neither the enhanced mucus thickness found during clearance in the WT mice, nor the increased mucus thickness observed in the IFN-$\gamma^{-/-}$ mice during infection could be explained by changes in mucin mRNA level. Therefore, it is important to combine mRNA measurement with studies on other aspects of the mucin glycoprotein life cycle.

The results from infection of the WT mice with *C. rodentium* showed mucus transcription and secretion are dynamically altered, and clearance of the infection coincides with the reformation of the organized inner mucus layer and the increase of mucus thickness. This increase in mucus layer suggested the involvement of mucins in removing the pathogens, which is in line with previous work that described the role of mucus in the removal of nematode infections [206,207]. In addition, during clearance of *C. rodentium* we detected an increase in ion channel activity compared to the highpoint of infection, when mucus thickness was decreased. The change in ionic composition was also detected in our mRNA and proteomics experiments. These results are further supported by the outcome of the study on the IFN-$\gamma^{-/-}$ mice that showed during the highpoint of infection, that these mice had a thicker mucus layer which again coincided with more activation of ion channels. As ion channels and their secreted Ca^{2+} ions and bicarbonate are suggested to be important to the packing and release of mucins [24-27], we speculate that the change in ion channel activity during clearance is to facilitate mucin release.

Furthermore, as discussed extensively in the Introduction, current knowledge indicates the effect of cytokines on goblet cell differentiation and the production rate

of mucins [50,56,68,74,75,77,78,80]. Therefore, we analyzed the mRNA of different cytokines involved in Th1/Th2 response in IFN-$\gamma^{-/-}$ and WT mice and detected a tendency toward the Th2 response in the absence of IFN-γ. The Th2 cytokine profile of the IFN-$\gamma^{-/-}$ mice coincided with the thicker mucus layer in these mice compared to *C. rodentium* infected WT mice. The change of cytokine environment toward a Th2 response is also detected in the mRNA analysis of cytokines in WT mice during the clearance of *C. rodentium* infection, when the mucus layer thickness is increased. In addition, the outcome of the treatment of *in vitro* mucosal surfaces with IL-4 and IL-13, that are cytokines involved in Th2 response, demonstrated an increase in the engorgement of the surface goblet cells and mucin synthesis, whereas Th1 cytokines had an inhibitory effect. The increase of Muc2 and Muc3 mRNA level and goblet cell hyperplasia in response to Th2 cytokines has been shown in the murine intestinal parasitic infections [78,79]. However, in the parasitic infection, the IL-13 cytokine was involved in induction of the mucin synthesis [78,79], while our experiments both *in vivo* and *in vitro* demonstrated a prominent effect of IL-4 in stimulation of mucin synthesis and turnover.

Changes in mucin rate and turnover in response to cytokine environment and infection

Changes in mucin synthesis and turnover in response to stimulators have been described in previous studies. The methods used are mainly based on PAS/alcian blue staining, immunohistochemical staining, measurement of the electrical parameters of the epithelial surface, and in some studies, radioactive labelling to quantify the freshly produced and secreted mucins [68,208-210]. In the present work, in addition to the common methods, we used a non-radioactive metabolic incorporation of acetylated GalNAz to the newly synthesized mucin. This method enabled us to follow the movement of the GalNAz labeled mucin from the time of translocation to the supra nuclear area of the cells and their release into the mucus layer, both *in vivo* and *in vitro*. The results from the stomach of non-infected mice and the intestinal *in vitro* model, along with the previous results from the murine distal colon [202] indicated that mucin synthesis and turnover is tissue and cell specific, and the renewal of mucin is faster in the intestine compared to the stomach. Furthermore, the results from the treatment of the *in vitro* mucosal surfaces with different cytokines indicated a reduction in rate and turnover of mucin in response to cytokines involved in Th1 response, whereas Th2 response cytokines increased the mucin synthesis and turnover. This was in line with a previously published experiment using an allergic model, where Th2 cells were intravenously transferred into mice, resulting in high levels of IL-4 and increased levels of Alcian blue/PAS stained mucins in the tissue [211]. Conversely, transfer of twice the number of Th1

along with Th2 cells (2:1), considerably decreased the amount of Alcian blue/PAS stained mucins [212]. However, our results from the infection of these treated cells with *C. rodentium*/ETEC demonstrated that bacterial invasion can overcome the effect of Th1 cytokines and induce the mucin synthesis and turnover in the cells treated with these cytokines, and accelerate the stimulation of cells treated with Th2 cytokines. Even non-treated cells showed an increase in rate and turnover of mucins in response to infection with *C. rodentium*/ETEC. In contrast, infection with *H. pylori* in mice reduced the rate of mucin synthesis and turnover mainly in the antrum.

In general, when comparing the results of infection studies with *H. pylori*, *C. rodentium* and ETEC presented in this thesis, it is important to consider the differences between *in vitro* and *in vivo* environment, bacterial density and duration of infection. As shown previously, during *in vivo* infections, in addition to the effect of microbial products and individual cytokines used in our *in vitro* study, a range of environmental stimuli can alter the rate of mucin release, including cholinergic stimuli, lipopolysaccharide, bile salts, nucleotides, nitric oxide, vasoactive intestinal peptides and neutrophil elastase [50,51,54-56,213,214]. In addition, effect of duration of infection on changes of mucins is detected during *in vitro* studies. For example, short time incubation of a human gastric mucosa biopsy segment with lipopolysaccharide from *H. pylori* resulted in rapid mucin discharged, whereas prolonged incubation led to a concentration-dependent decrease in mucin synthesis and secretion [215]. Similar results were observed during *in vitro* infection of the intestinal goblet cells by viable *H. pylori* [215].

Changes of mucin and mucus layer, during infection in gastric mucosa

We have also studied the effect of *H. pylori* infection on the murine gastric mucosa, which demonstrated a decrease in Muc1 during early colonization and chronic infection. The Muc5ac levels also suggested a decrease, albeit it was not statistically significant. However, due to the lack of a proper preservation method and probable differences in the properties of the inner adherent layer between stomach and colon [6,216], we did not succeed in preserving the mucus layer in the stomach. It has been shown that preserving colon along with luminal material helps in keeping the mucus layer intact [217]. Nevertheless, despite our effort to preserve the whole stomach with the luminal material, most of the inner mucus layer was lost, and the remains were only detectable as patches on the epithelial surface. In addition, culture of gastric cells with the different methods did not improve the morphology of cells as detected in intestinal cell lines. Only MKN7 cells could produce an adherent polarized layer, but lacked the adherent mucus layer. In summary, these

methodological problems are obstacles in the studies of the gastric mucus layer and mucins during infection. To improve the understanding of the interaction between gastric pathogens and mucosal surfaces, there is a need to improve the existing methods or providing new methods compatible to the gastric mucosal surface.

CONCLUSIONS

The main focus of this thesis was to investigate the changes in mucins and the mucus layer in the gastrointestinal tract during infection with the gastrointestinal pathogens *C. rodentium* (a mouse model for intestinal A/E pathogens), ETEC and *H. pylori*. To be able to compare the results from murine studies to the effect of infection in humans, we needed an *in vitro* mucosal surface to have the most resemblance to the *in vivo* environment. Therefore, we developed a method of culture to create *in vitro* model suitable for studies of host-pathogen interactions at the mucosal surface. Our main findings were:

- The semi-wet interface culture in combination with mechanical stimulation and DAPT treatment caused HT29 MTX-P8, HT29 MTX-E12, LS513 and to some extent, Caco-2 and T84 intestinal cells to polarize, form functional tight junctions and produce an adherent mucus layer.

- *H. pylori* colonization in the mucus niche of the murine stomach *in vivo* leads to decreased mucin production and secretion rate and decreased levels of Muc1 in the mucosa.

- During self-limiting infection of *C. rodentium* in WT mice, mucus transcription and secretion are dynamically altered in response to the infection and the clearance of the infection coincides with the reformation of the organized inner mucus layer and an increased mucus thickness.

- The increase in mucus thickness during *C. rodentium* clearance coincided with altered ion channel activities.

- Changes in the cytokine environment *in vivo* has a huge effect on mucus thickness, as infection of IFN-$\gamma^{-/-}$ mice with *C. rodentium* resulted in a vastly enhanced mucus thickness compared to the WT animals.

- Both the cytokine profile and the pathogen species affect the production rate and turnover of mucins, and the presence of the Th2 cytokines accelerated the process of mucin synthesis.

ADDITIONAL BIBLOGRAPHY

Skoog EC, Sjoling A, **Navabi N**, Holgersson J, Lundin SB, et al. (2012) **Human gastric mucins differently regulate Helicobacter pylori proliferation, gene expression and interactions with host cells.** PLoS One 7: e36378.

ACKNOWLEDGEMENT

This thesis is a result of a quest for knowledge that started from the first day of the school, and I would like to thank each and every person who guided, encouraged or inspired me during these years. My special gratitude is to:

My main supervisor **Sara Linden**, I admire your knowledge, enthusiasm and I am grateful that you believed in me. Thank you for your never ending support. You taught me to think and work independently, encouraged my ideas and motivated me.

My co-supervisors **Gunnar** and **Henrik,** thank you for your help and support.

All the past and present members of the MIC team for contributing to such a nice working environment.

Emma, for being a wonderful friend and colleague, and a great company during our scientific trips. For all the guidance and help with the PhD rules during these years. I wish you a lot of success in the rest of your career.

Harvey, you are always ready to share your knowledge and I learned a lot from you. I am especially grateful that you take the time to read through this thesis.

Pushpa, for your helps and support during these years, and for being a great friend.

Debashish, for all the talks during these years, you are always ready to help everyone.

Macarena, Medea and Jonas, for your friendship and kindness. **Elsa, Arpan, Omid** and **Anna**.

A special thanks to **Åsa Sjöling,** you were always ready to teach me and answer my questions. I had your support from the day I started my Master thesis and could always count on your help.

Sukanya and **Malin Johansson**, for your contributions to the work and your advice on the methods.

All members of the mucin biology, Susann and Niclas's group you have all provided a great and friendly atmosphere and it has been a pleasure working with all of you. I would like to especially thank **Jenny**, for always being ready to answer my questions about the Ussing method, and **Karin** for all your helps. **Sara Flowers,** I am so grateful for your moral supports and kindness, especially during these last months.

The people in **the department of microbiology and immunology** for letting me use the equipment and all their helps. Specially:

Ali Harandi, my Master thesis supervisor, for introducing me to the research world and for his endless support. Also to the members of the group**, Josefine, Madelene** and **Karolina,** you are the best friends and co-workers. I learned a lot of methods and lab routines from you.

Linda Löfbom, for being a great friend, I could always count on your help and support. Thank you.

Margareta and Anneli, you were always ready to answer my questions and guided me a lot during the *H. pylori* infection.

Julia and Maria from the center for cellular imaging for the instructions during work with confocal microscope.

I would also like to thank the **Tony Hollingsworth** and all the members of his group specially **Tom, Paul and Jim** for scientific discussions, your collaboration and your hospitality during the time I spent in your lab.

The organizers of Forum GIMIICum, you managed to broader my vision with your wonderful courses. Also to members of the Forum for the grate scientific discussions, and the fun times we had during our gatherings.

My **mom and dad** for teaching me to work hard and be independent, and **my sister** for the constant encouragements.

A special thank to my son **Sohrab,** for your patience during these years. It was not easy to have a mom who was always busy studying, but you understood and gave me strength.

A huge thank to my wonderful husband. **Mansur** without you and your support I could never finish this thesis. You were always there, listening to me, supporting me, and encouraging me. Thank you for your patience, especially during these last months.

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